

KAIDO SIIMON

Electrospun gelatin
cross-linked by glucose



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PUBLICATIONS

The current dissertation is based on the following publications:

- I. Kaido Siimon, Paula Reemann, Annika Pöder, Martin Pook, Triin Kangur, Külli Kingo, Viljar Jaks, Uno Mäeorg, Martin Järvekülg. Effect of glucose content on thermally cross-linked fibrous gelatin scaffolds for tissue engineering. *Materials Science & Engineering C* 2014; 42: 538–545. doi: 10.1016/j.msec.2014.05.075
- II. Kaido Siimon, Hele Siimon, Martin Järvekülg. Mechanical characterization of electrospun gelatin scaffolds cross-linked by glucose. *Journal of Materials Science: Materials in Medicine* 2015; 26:37. doi: 10.1007/s10856-014-5375-1
- III. Kaido Siimon, Karol Möisavaal, Hele Siimon, Martin Järvekülg. Increasing mechanical strength of electrospun gelatin nanofibres by the addition of aluminium potassium sulphate. *Journal of Applied Polymer Science* 2015; 42431. doi: 10.1002/app.42431

The author of the current dissertation, closely cooperating with co-authors of the above-mentioned articles, was responsible for designing the study, conducting experiments, analysing results, writing the manuscripts (except for sections 2.5–2.9 and 3.4 in publication I), and serves as the corresponding author for all the above-mentioned publications. The articles are referred to using Roman numerals in the text.

1. PREFACE

The initial purpose of our work concerning electrospun gelatin was developing transplantable tissue engineering scaffolds for skin regeneration, focusing on using natural, non-toxic substances and simple, cost-effective methods. Such a scaffold is basically a thin mesh, which is used to cover a wound to promote healing. The first step of the scaffold preparation process is preparing an artificial extracellular matrix on which the cells can be cultured. Gelatin was chosen to be the main component of the artificial extracellular matrix, because it is a protein derived by hydrolysis from collagen, a naturally occurring protein most abundant in connective tissue such as skin [1, 2]. However, gelatin is cheaper than collagen and easier to process. Both collagen and gelatin have been considered as materials for tissue engineering scaffolds and share many advantages (biocompatibility, biodegradability, non-toxicity) as well as disadvantages (nonhomogeneous composition and inconsistent physicochemical properties) [3].

Natural extracellular matrix consists mainly of bundled fibres. In order to mimic this structure to a certain degree, fibrous gelatin scaffolds were prepared using the electrospinning technique. However, electrospun gelatin is water-soluble and must be cross-linked to make it insoluble, since cells are cultured in an aqueous environment. Previously, mostly expensive and more or less toxic chemical cross-linking agents have been used to treat fibrous gelatin. The current dissertation is based on the discovery that electrospun gelatin fibres can be thermally cross-linked by glucose. The discovery brought up several questions – how much glucose should be added in order to achieve desired extent of cross-linking, how does cross-linking by glucose affect the properties of the material, can the fabrics be used for cell culture applications, etc. The abovementioned articles provide answers to many of such questions. The results are summarised in the current dissertation, providing additional information where necessary.

A large part of the dissertation is dedicated to mechanical properties of electrospun gelatin cross-linked by glucose because of the following reasons. Firstly, mechanical behaviour plays a key role in many applications, including biomedicine, filtering, protective clothing and fabrics. However, low mechanical strength has been limiting the use of fibrous gelatin in these applications, and it is vital to find ways to increase the strength of the material. Secondly, mechanical properties of biopolymer fibres are influenced by numerous factors including the quality of the used gelatin, fabrication process parameters, solvent used to prepare the electrospinning solution, humidity, fibre size etc. As a result of all these different factors influencing the properties of electrospun materials, the reported elastic moduli of electrospun gelatin treated with miscellaneous cross-linkers vary to a very large degree and it is hard to reproduce the results presented in literature and compare it to one's own results, which creates a lot of confusion. Therefore, it is vital to study mechanical behaviour of the material

in great detail in order to be able to repeatedly prepare fibres with similar mechanical properties.

The main purpose of the study was to prepare and characterize electrospun gelatin cross-linked thermally by glucose, and to develop strategies for gaining control over its mechanical properties.

2. INTRODUCTION

2.1 Electrospun gelatin

Interest in natural fibrous materials is continuously increasing. The fibrous structure that can be achieved by electrospinning is important in various applications in the biomedical field, cosmetics, energy storage, filtration, protective clothing, sensors, space applications, optoelectronics, and in composite materials [4, 5, 6]. Electrospun gelatin has been studied mainly for biomedical applications, including wound healing, tissue engineering blood vessels, heart valves, bone, cornea, regeneration of periodontal tissue, nerve tissue, controlled release [7, 8, 9, 10, 11, 12, 13, 14].

Several methods can be used to fabricate fibrous materials, including phase separation, self-assembly, surface patterning, interfacial complexation, wet-, bio-, melt- and microfluidic spinning and electrospinning, the latter being a well-established, simple, cost-effective and flexible technique that takes advantage of electrostatic forces to produce polymer fibres [15, 16, 17, 18]. Gelatin can be electrospun from solvents like 1,1,1,3,3,3-hexafluoro-2-propanol, 2,2,2-trifluoroethanol and solvent systems like acetic acid/ethyl acetate/water or aqueous formic acid [19, 20, 21, 22]. It has been reported that gelatin can be electrospun from water above 36°C [23, 24]. At room temperature, gelatin can be electrospun from acetic acid or its water solutions, as it is done in the current work, but fibres are produced only when the concentration of acetic acid is sufficiently high [25].

Gelatin has been co-electrospun with several other polymers including elastin, chitosan, silk, hyaluronic acid, fibrinogen, poly(ϵ -caprolactone), polyurethane, poly(vinyl alcohol) [26, 27, 28, 29, 30, 31, 32, 33]. Several additives can be used to alter the properties of electrospun gelatin, including nanoparticles, herbal extracts, salts, and so forth [III, 34, 35, 36].

In conclusion, electrospun gelatin can easily be prepared using only natural substances and easy, cost-effective methods. Such fibrous gelatin is also relatively cheap, extremely biocompatible and biodegradable material. However, electrospun gelatin is water-soluble and mechanically weak. Overcoming these disadvantages is discussed in the following chapters.

2.2 Cross-linking of electrospun gelatin

Cross-linking is a widely used strategy for making electrospun gelatin insoluble and to increase its strength. Cross-linking decreases solubility by creating chemical bonds between polymer chains and has been shown to increase mechanical strength of electrospun gelatin [37, 38]. Cross-linking of gelatin fibres can be carried out using chemical, physical, enzymatic [39] or combined methods. Physical cross-linking methods include thermal, plasma and ultra-violet treatment [40, 41, 42, 43, 44]. However, physical cross-linking methods

generally result in a low degree of cross-linking, which can be sufficient to make gelatin insoluble in water, but the use of chemical cross-linking agents is necessary to achieve considerable changes in mechanical properties of the material. Additionally, chemically cross-linked electrospun gelatin has been reported to be better suited for tissue engineering applications compared to thermally cross-linked electrospun gelatin [45]. Numerous cross-linking agents have been used to treat electrospun gelatin, including glutaraldehyde, terephthalaldehyde, dextran aldehyde, genipin, oxidized sucrose, caffeic acid, tannic acid, procyanidine, proanthocyanidin [46, 47, 48, 49, 50, 51, 52, 53]. However, most chemical cross-linkers are costly and potentially toxic. Cytotoxicity of a cross-linking agent depends on the type and amount of cross-linking agent and the cross-linking process [54]. The latter is often quite hard to control, which makes the use of potentially toxic cross-linking agents more or less dangerous in biomedical applications.

Glucose has many advantages (biocompatibility, non-toxicity, cost-effectiveness) compared to widely used chemical cross-linking agents such as dialdehydes. However, glucose alone has been reported to be a relatively ineffective cross-linking agent [55], mostly because the formation of glucose cross-links requires glucose molecules to be in their linear form [56], but at normal conditions glucose molecules are in the closed ring form [57]. Nevertheless, it is known that amino acids and reducing sugars react at high temperatures through the Maillard reaction pathway and that the presence of sugars can alter the conformation and interactions of proteins, having an effect on the cross-linking process [58, 59, 60]. As described in the current work [I–III], electrospun gelatin can be thermally cross-linked by glucose simply by placing the glucose-containing as-electrospun fabric into a preheated oven at about 170°C, ensuring proper cross-linking and preserving the nanofibrous structure of the fabric.

2.3 Mechanical properties of electrospun gelatin

Mechanical behaviour of materials plays a key role in several applications, including biomedicine. For example, stiffness of artificial substrate is known to influence cell growth, proliferation and differentiation in tissue engineering applications [61, 62]. Low mechanical strength has been limiting the use of fibrous gelatin.

Mechanical strength of electrospun gelatin depends on several factors. Properties of gelatin depend on the source of collagen, age of animal, type of collagen, type of conversion of collagen to gelatin (acidic or basic hydrolysis) [63]. The properties of electrospun materials are affected by fabrication process parameters, fibre orientation, orientation of macromolecules inside the fibres, mechanical anisotropy, humidity, existence of beads on the fibre surface [64, 65, 66, 67]. Furthermore, the stress-strain behaviour of electrospun fibres has been found to be different from bulk material from which it was prepared [68],

making evaluation of fibre properties even harder. Mechanical strength of cross-linked polymers is determined by the type and amount of the cross-linking agent, the chosen cross-linking method, and the extent of cross-linking. Exact characterization of mechanical properties of electrospun bio-polymeric fibres remains challenging, mainly because it is difficult to take all these factors into account at the same time.

A variety of approaches have been applied towards mechanical characterization of nanofibres and fabrics by employing nanoindentation, bending tests, resonance frequency measurements and tensile tests [69, 70, 71]. Mechanical properties of fibrous materials have been modelled at micro- and macro-scale in various ways mostly based on bending and tensile tests [72, 73, 74, 75].

The effect of fibre size on mechanical strength of fibrous bio-polymeric materials is rarely considered. Some authors have reported that the strength of electrospun polymers increases with decreasing fibre diameter [76, 77]. Additionally, it is known that various surface effects can cause the strength of inorganic nanofibres to increase with decreasing fibre diameter [78, 79, 80]. The strength of cross-linked gelatin fibres is greatly affected by the presence of intra-fibrous hydrogen bonds and cross-links, which's concentration is proportional to fibre diameter, which contributes more to the strength of larger fibres.

In the current study, the efforts made to increase mechanical strength of electrospun gelatin are based on the following speculations. Firstly, it is known that physical cross-linking techniques such as thermal treatment result in a very low extent of cross-linking. In order to significantly increase mechanical strength of electrospun gelatin, it is necessary to achieve a higher extent of cross-linking. This can be achieved by using glucose as a cross-linking agent. In order to do that, the fabrics were heated to about 170°C. As mentioned above, amino acids like gelatin and reducing sugars like glucose react at high temperatures through the Maillard reaction pathway. The Maillard reaction involves mainly certain amino groups of gelatin (ϵ -amino groups of lysine and α -amino groups of terminal amino acids) [81]. However, lysine content in gelatin is quite low (4–5% of dry ash-free protein) [82, 83]. This is one of the aspects limiting the maximum extent of cross-linking that can be achieved by using glucose as a cross-linking agent. In order to further increase mechanical strength of the fibres, other functional groups must be involved in the cross-linking process.

The type A gelatin used in the current work contains 78–80 mmol of free carboxyl groups per 100 g of protein (according to the product information sheet). It is known that aluminium forms cationic basic ions, capable of reacting with collagen and the reaction sites for Al(III) are the carboxyl groups of collagen [84]. Aluminium potassium sulphate, a natively occurring salt, has long been used in leather tanning to enhance its mechanical properties and make it more durable [85]. This suggests that the addition of $\text{AlK}(\text{SO}_4)_2$ could also lead to an increase in mechanical strength of gelatin-based materials by initiating additional cross-linking mechanism involving carboxyl groups of gelatin. However, in case of leather tanning the additive is used to treat existing bundled

fibres, whereas in case of artificially prepared fibres the additive can be placed directly inside the fibres. Therefore, the effect of the additive can be expected to be stronger and last longer than in tanning applications.

In the current dissertation, an increase in mechanical strength of electrospun gelatin is achieved by combining thermal treatment with glucose- and alum-initiated cross-linking. In addition to the effects of cross-linking, other aspects influencing the strength of the fabrics are analysed, including the effect of aging of the electrospinning solution.

3. MATERIALS AND METHODS

3.1 Preparation of fibrous gelatin

Gelatin type A from porcine skin, glucose, and glacial acetic acid were purchased from Sigma-Aldrich. Aluminium potassium sulphate dodecahydrate was purchased from Lach-Ner. All the process parameters described below were obtained as a result of process optimization and preliminary experiments.

Gelatin was mixed with glucose so that the mixtures contained up to 30% glucose. In order to study the effect of aluminium potassium sulphate on mechanical strength of electrospun gelatin cross-linked by glucose, aluminium potassium sulphate dodecahydrate was added to a mixture containing 10% glucose so that the mixtures contained up to 17% alum. The mixtures were dissolved in 10 M aqueous acetic acid to obtain 19–25% solutions.

Gelatin fabrics were prepared from these solutions by electrospinning (Figure 1). A syringe containing the solution was mounted on a New Era Pump Systems NE-511 pump operating at 6–10 $\mu\text{l}/\text{min}$. High voltage (17.5 kV) was applied to a blunted metallic syringe needle using Heinzinger LNC 30000 high voltage power supply. A grounded target (plate collector) was placed at 12–15 cm distance from the needle tip. Fibrous meshes were collected from the target after electrospinning for 15–30 min and stored for further treatment. Fibre diameter was manipulated by varying the concentration of the electrospinning solution – the higher the concentration, the larger the fibres.

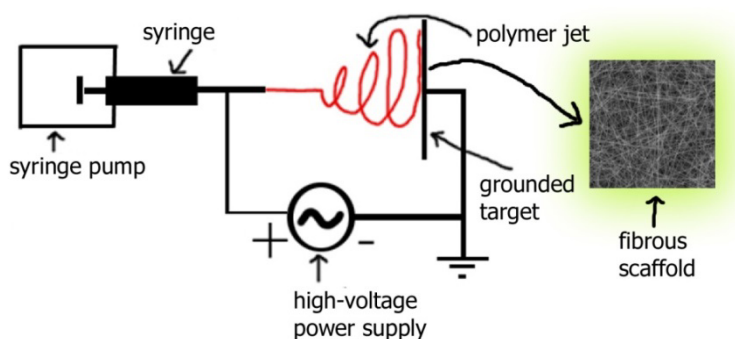


Figure 1 Schematic representation of the used electrospinning setup comprising a syringe pump filled with polymer solution, a high voltage power supply and a grounded target. A droplet forms on the needle tip as the solution is pushed out of the syringe. The droplet becomes charged when high voltage is applied to a metallic syringe needle and a polymer jet is pulled towards the grounded target by electrostatic forces, resulting in the formation of polymer fibres, which deposit on the grounded target one by one and layer by layer, forming a fabric.

In order to avoid thermal degradation of gelatin while ensuring proper cross-linking and to operate above melting point and caramelization temperature of glucose, the fabrics were cross-linked by placing them in a preheated oven for 3 h at 170–175°C (Figure 2). In order to ensure proper cross-linking while maintaining structural integrity of the material, the oven must be preheated to this temperature before inserting the fabrics. Pieces of the material were removed from the oven after various times from 5 min to 3 h and analysed to monitor the cross-linking process.

3.2 Scanning electron microscopy (SEM)

Samples were dried using Leica EM CPD300 critical point drier, covered with 5–7 nm layer of gold using Polaron SC7640 sputter coater and analysed by SEM (Tescan Vega). Brightness and contrast of the images was adjusted and the colours of some images were inverted to make them easier to analyse using Adobe Photoshop CS2 software. Fibre diameters were measured from 21000 times magnified images. Fibre diameter distribution graphs were constructed by measuring the number of fibres with diameters ± 30 nm from a certain fibre diameter. Such technique allows obtaining smooth histograms and facilitates visual appraisal of the graphs.

Porosity of the meshes was determined in the following way. Firstly, SEM images were divided into layers using colour codes of pixels at the centre of the fibres. Secondly, the diameters and length of the fibres present in the layer were measured. Next, porosity of a layer P was calculated from the equation

$$P = 100\% * \frac{\sum_i \pi (\frac{d_i}{2})^2 L_i}{V}$$
, where d_i is the diameter of the i -th fibre, L_i is the length of the i -th fibre, and V is the volume of the layer under consideration (depth of the layers was evaluated using the measured fibre diameters). Lastly, porosity of the mesh was determined by averaging the porosities of the layers determined from several images.

3.3 Fourier transform infrared microscopy (FTIR)

After drying in Leica EM CPD300 critical point drier, FTIR spectra between 400 cm^{-1} and 4000 cm^{-1} were recorded using Bruker Vertex 70 spectrometer equipped with an attenuated total reflection (ATR) accessory. The ATR spectra were converted to absorbance spectra after baseline correction and normalized to a constant penetration depth using OPUS software.

Absolute values of recorded absorbances were not reproducible due to the fibrous structure of the material and small fibre diameter, which did not allow achieving perfect contact between the fabric and ATR crystal. In order to be able to reliably compare infrared spectra of samples with different composition, relative absorbances were calculated by dividing absorbance of the peak in question by the sum of absorbances of all relevant peaks detected in this particular spectrum.

3.4 Characterization of mechanical properties of electrospun gelatin

3.4.1 Tensile test

Electrospun fabrics were cut into rectangular segments and their dimensions and weight were measured. Smaller, approximately 3 cm long and 1.5 cm wide samples were cut out from these fabrics and their thickness was measured using a calliper. Tensile test was carried out using a self-built tensile testing station (10 μ m distance measurement accuracy) equipped with Sauter FH100 force sensor. Samples were fixed between clamps and pulled at 5–10 mm/h, elongation and force were recorded. The fabrics were photographed during tensile test and the changes in their dimensions at given elongation were evaluated using Adobe Photoshop CS2 software.

3.4.2 Modelling

Modelling of fibre deposition

The first step of tensile test simulation was creating a virtual fibrous scaffold. This was done by simulating fibre deposition during electrospinning. The total length of fibres per volume in the scaffold was calculated using the dimensions, weight, porosity and fibre diameter measured beforehand. Fibres were placed randomly one by one and layer by layer to form a virtual scaffold until the calculated total fibre length was reached. Based on SEM data, the fibres were assumed to be straight and remain straight during tensile test. It was observed from SEM images taken after failure that fibres in the scaffolds tested before cross-linking were pulled straight next to the ripping point (Figure 2a). The fibres that were further away from the ripping point also became noticeably oriented in the direction of the applied force. Scaffolds tested after cross-linking revealed very little fibre orientation right next to the ripping point. Furthermore, no increase in fibre orientation was observed by SEM when the meshes were pulled only in the elastic region of the strain and removed from the tensile testing machine before ripping (Figure 2b). This confirms the assumption that cross-links form between individual fibres as well as between gelatin molecules in each fibre and suggests that in the elastic region where deformation is small the fibres do not slide on each other and friction does not have to be taken into account when describing tensile testing of cross-linked scaffolds.

The behaviour of the scaffolds during tensile test depends on the interactions between the fibres. Non-cross-linked fibres are relatively independent of each other, the interactions between fibre segments that are in contact are weak and all the fibres tend to change their orientation in the direction of the applied force during tensile test. Friction alone causes some resistance to fibre reorientation. In case of cross-linked fibres chemical bonds have formed not only between polymer molecules inside each fibre, but also at the interceptions of fibres. These chemical bonds connecting individual fibres make the mesh structure

rigid and reorientation of a fibre caused by the applied force becomes dependent on reorientation of other fibres.

Tensile test was simulated for cross-linked scaffolds only, because non-cross-linked scaffolds are water-soluble and thus not usable in practical applications. Comparing SEM images (Figure 2c) with virtual meshes (Figure 2d) constructed in the manner described above confirmed the necessary similarity between the virtual and the real scaffold.

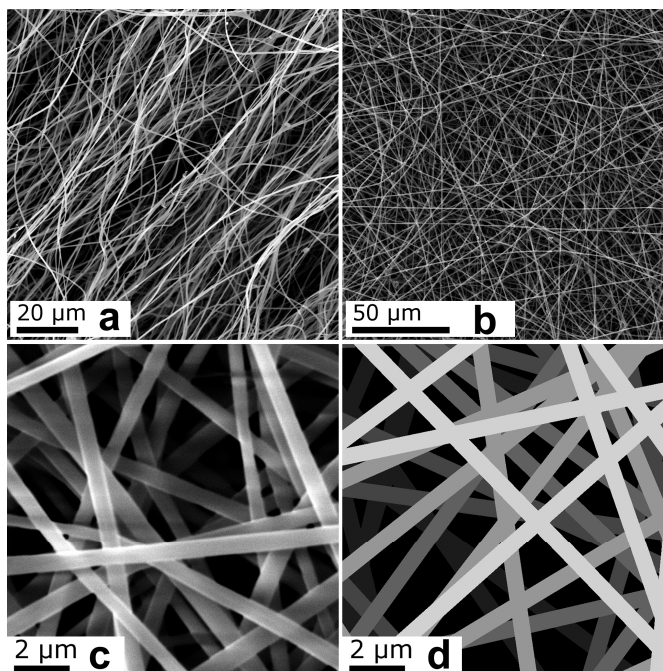


Figure 2 SEM images of (a) a non-cross-linked gelatin fabric after tensile test 100 μm from the ripping point and (b) a cross-linked gelatin fabric after tensile test. Both in case of cross-linked and non-crosslinked samples, the fibres were randomly oriented before tensile test. During tensile test, the non-cross-linked fibres were reoriented in the direction of the applied force, whereas cross-linked fibres were not. (c) 21000 times magnified SEM image of a gelatin scaffold and (d) the virtual 3D scaffold constructed by the model; brightness of the fibres expresses the depth of the scaffold – deeper layers are darker.

Modelling of tensile test

Tensile test was simulated using virtual scaffolds constructed as described above. The goal of the simulation was to obtain the elastic region of stress-strain diagram. Fibre diameter d , elastic modulus E , a coefficient G (the value of G is proportional to shear modulus) were used as constants characterizing the material. Fibre material was considered to be brittle so that Hooke's law was valid in the whole simulated region of the stress-strain diagram. At each simulation step, a small increase was

given to the length of the sample. This causes stress in the fibres. The total tensile force is equal to the sum of the forces resulting from all fibres. Decrease in mesh width (neck formation) at larger elongations is related to the strength of interactions between the fibres. It was observed experimentally that the change in shape (forming of neck due to decreased mesh width) is related to glucose concentration in the fibres. The more glucose the scaffolds contained (and therefore the higher the extent of cross-linking) the smaller was the decrease of sample width. Scaffolds containing 15% glucose did not form any neck during tensile testing. During simulation the shape change of a piece of scaffold was determined at each step. In this work we assumed that the studied piece of scaffold remains rectangular during the test. This assumption is strictly valid for central parts of the mesh. However, if the dimensions of the scaffold piece are chosen to be small and tensile test is simulated only in the elastic region where deformation is also small, then the approximation that the scaffold is rectangular describes tensile testing accurately enough even in case of neck-formation.

Figure 3 describes the scheme of the simulation. y -axis is chosen so that it coincides with the direction of the applied force (and scaffold elongation). Each step of the simulation gives one point to the force-elongation diagram and consists of the following parts. Firstly, a small increase is given to the scaffold length, causing relative elongation of the scaffold ε_y , while the width of the scaffold is kept unchanged. This induces elongation and stress in each fibre depending on the angle α_i between the fibre and x -axis, which in return causes normal stress σ_i in i -th fibre. Normal stress was calculated using the equation $\sigma_i = E \varepsilon_i$, where E is the expected elastic modulus, ε_i is the relative elongation of the i -th fibre and α_i is the angle between the i -th fibre and x -axis. Increase in length of the scaffold causes a change $\Delta\alpha_i$ in the angle α_i . The fibres tend to be oriented towards the y -axis during tensile testing. It was taken into account that the fibres are cross-linked and relatively strong chemical bonds exist at the interceptions of individual fibres. Thus the fibres do not slide on one another and the structure is relatively rigid. Therefore torsion arises around these interceptions. Generally, the shear stress (tangential stress) τ_i in a fibre at torsion is proportional to the torsion angle. We calculated tangential stress using the equation $\tau_i = G \Delta\alpha_i$, where G is a coefficient characteristic to the fibre material and proportional to its shear modulus.

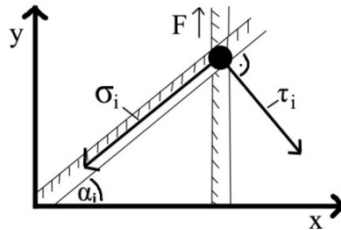


Figure 3 The conceptual scheme of the tensile test simulation. Applied force causes elongation of a sample, which in return causes normal stress (σ_i) and tangential stress (τ_i) in the fibres.

Evaluation of the coefficient G is based on the following speculations. The ratio of the elastic modulus and the shear modulus depends on the Poisson coefficient, i.e. the ratio of the relative decrease of the specimen width to the relative increase of its length. The Poisson coefficient determines the change in volume of a material during tension, and the relative change of the surface area in our tensile test. Therefore we expected the E/G ratio to be connected to the relative change in the dimensions and shape of the scaffold during testing. Using the sample dimensions evaluated from the images taken during experimental tensile testing and comparing them to the dimensions calculated from the simulations at various E/G ratios, it was found that the values of E/G varied between 1.0 (for samples containing 15% glucose) and 1.2 (for samples containing no glucose).

The second part of this simulation step takes advantage of the calculated stresses σ_i and τ_i to determine the change in mesh width. We calculated x - and y -components of normal and tangential stresses by $\sigma_{ix} = \sigma_i \cos(\alpha_i)$, $\sigma_{iy} = \sigma_i \sin(\alpha_i)$, $\tau_{ix} = \tau_i \sin(\alpha_i)$, and $\tau_{iy} = \tau_i \cos(\alpha_i)$. The y -components of σ_i and τ_i add up and their sum is balanced by the applied force. The direction of the x -components of σ_i and τ_i is determined by the angle α_i . The x -components σ_{ix} and τ_{ix} of each fibre are directed at opposite directions. The difference between their values is related to the change in fibre orientation and the decrease in scaffold width. Additionally, it gives rise to neck formation during testing. It must be mentioned that the sum of forces resulting from the x -components of the stresses in all fibres is equal to zero, whereas the difference between σ_{ix} and τ_{ix} in each individual fibre contributes to neck formation. The sum of y -components of the stresses in all the fibres is proportional to the applied force, $F_y = (\pi d^2/4) \sum_i (\sigma_{iy} + \tau_{iy})$. The force causing the narrowing of the scaffold is caused by the differences in stress components, $F_x = (\pi d^2/4) \sum_i |\sigma_{ix} - \tau_{ix}|$. Relative decrease of the mesh width, ε_x , follows from the equation $\frac{\varepsilon_x}{\varepsilon_y} = \frac{F_x}{F_y}$, which gives

$$\varepsilon_x = \varepsilon_y \cdot \frac{\sum_i |\sigma_i - \tau_i|}{\sum_i (\sigma_i + \tau_i)}.$$

Finally, the values of ε_y and ε_x were used to recalculate fibre lengths, stress in the fibres, and the total force F_y stretching the scaffold. The force directly causing elongation of the sample was calculated as the sum of forces determined for all fibres. In this way, tensile test was simulated by firstly calculating new dimensions of the sample, and secondly the force applied in the y -direction. So, step by step, the force-strain diagram was generated. Tensile test was simulated, adjusting the hypothetic elastic modulus, until the elastic region of experimental tensile test graph and the simulated tensile test graph overlapped. The elastic modulus of the fibre material was found for all the samples in this manner.

4. RESULTS

4.1 Preparation of electrospun gelatin

Mixtures of gelatin and glucose were found to be electrospinnable from 5 M and more concentrated aqueous acetic acid solutions, but the suitable range of electrospinning parameters became narrower at lower acetic acid concentrations. 10 M aqueous acetic acid was found to be suitable for electrospinning all the mixtures used in this work, allowing problem-free production of fibres in a wide range of process parameters. Glucose content had little effect on the suitable range of electrospinning parameters, which seemed to be determined mostly by the concentration of gelatin.

20–150 μm thick gelatin fabrics were obtained as a result of electrospinning. Smooth and uniformly structured fibres were produced at up to 30% glucose content. However, easy to handle fabric-like material was produced at up to 15% glucose content. At higher glucose concentrations the fabrics became stiff, brittle and broke easily after thermal treatment.

Density of the fabrics varied between 150 and 220 mg/cm^3 , mostly depending on composition and porosity of a fabric. Porosity of the fabrics determined using SEM images varied from 78% to 90% (average 82%). No correlation was found between fibre diameter and porosity of the fabrics. Porosity of electrospun gelatin is greatly influenced by randomness of the electrospinning process (Figure 4), which causes variabilities.

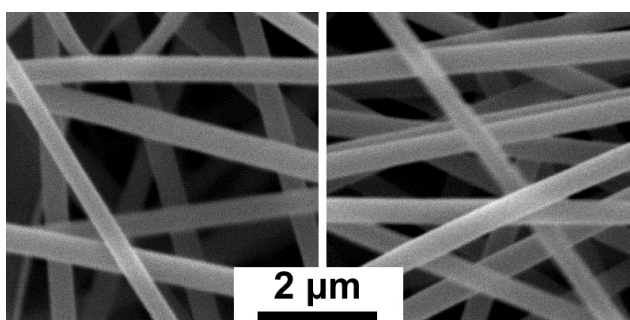


Figure 4 SEM images of samples prepared using the same preparation process parameters indicate that porosity of a fabric is greatly influenced by randomness of the electrospinning process.

Porosity of an electrospun mesh also has a strong impact on mechanical strength of the material. Mechanical strength of a fabric is inversely proportional to its porosity, leading to the conclusion that porosity must be taken into account in studies concerning the strength of electrospun materials. However, there's a lot of confusion concerning porosity of electrospun materials. Porosity is affected by the electrospinning process parameters [86]. It has been found

that porosity of an electrospun mesh is not dependent on fibre diameter, although porosities determined using various techniques vary greatly [87]. Additionally, porosity has been reported to both increase [88] and decrease [89] with increasing fibre diameter. In the current work, porosity was not found to be in correlation with fibre diameter.

4.2 Effect of concentration of the electrospinning solution on fibre diameter

Varying the concentration of the electrospinning solution (the amount of 10M acetic acid added to the gelatin-glucose mixture) between 19–25% allowed fabrication of gelatin meshes with average fibre diameters between 200–700 nm (Figure 5). A few samples had an average fibre diameter higher than 700 nm or lower than 200 nm. This is caused by hard to control instabilities of the electrospinning process like the size of the droplet that forms on the tip of the needle used in electrospinning.

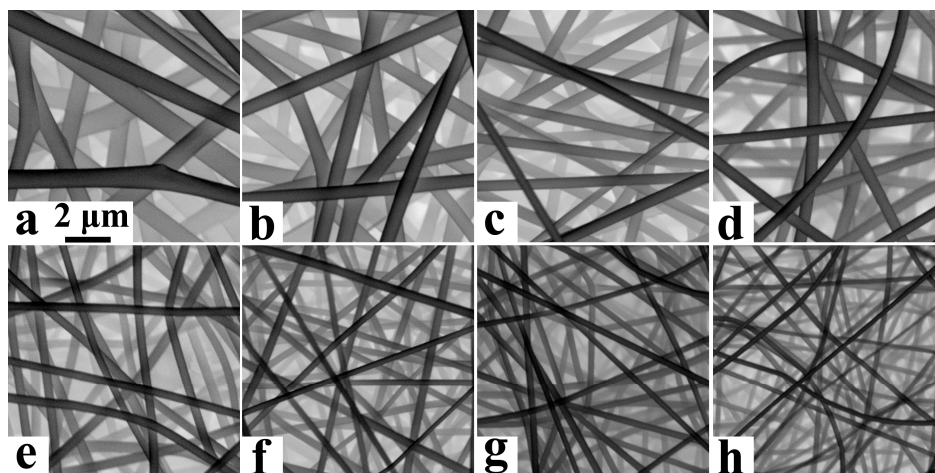


Figure 5 21000 times magnified SEM images of electrospun gelatin fabrics containing 10% glucose as a cross-linking agent prepared by varying the concentrations of the electrospinning solution; average fibre diameters: 784 nm (a), 594 nm (b), 472 nm (c), 444 nm (d), 352 nm (e), 288 nm (f), 259 nm (g), 188 nm (h). The colours of the original SEM images were inverted to improve visibility of the deeper layers.

The correlation between the concentration of the electrospinning solution and average fibre diameters is demonstrated in Figure 6. It is important to keep in mind that average fibre diameter is affected by several other factors in addition to the concentration of the electrospinning solution. For example, aging of the electrospinning solution has a strong effect on average fibre diameter, which is

considered in a following section of the current work. Therefore, in order to repeatedly prepare fabrics with similar average fibre diameters, all the parameters of the electrospinning process must be kept as constant as possible.

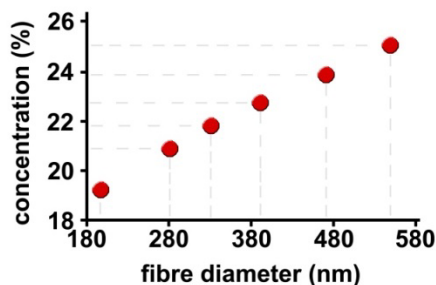


Figure 6 Dependence between the concentration of the electrospinning solution and average fibre diameter. Fibre diameter is determined largely by concentration of the electrospinning solution, although other factors like aging of the solution also have an effect on it.

Fibre diameters vary to some extent in case of all samples. However, the shape of the fibre diameter distribution graphs of samples with various average fibre diameters was largely similar (Figure 7), although the range of fibre diameters increased in case of samples consisting of larger fibres. Additionally, the number of abnormally small or large fibres increased as the average fibre diameters increased. This was probably caused by the used electrospinning setup – higher viscosity of more concentrated solutions caused the formation of a larger droplet on the needle tip, which in turn makes the electrospinning process less stable.

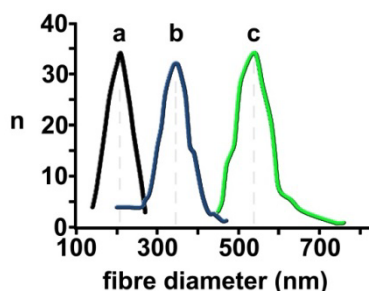


Figure 7 Fibre diameter distributions of samples with the following average fibre diameters: 197 nm (a), 332 nm (b), 551 nm (c)

4.3 Effect of aging of the electrospinning solution on fibre diameter

Viscosity of solutions of gelatin in aqueous acetic acid has been shown to decrease in time [90], which is most likely caused by protonation of gelatin. Solvent used in the current work (10M aqueous acetic acid) has a pH much lower than the isoelectric point of type A gelatin, leading to the conclusion that gelatin slowly becomes protonated, which in return causes a decrease in viscosity of the solution due to weakening of electrostatic attraction between gelatin molecules. Therefore, it is necessary to determine the effect of aging of the electrospinning solution on fibre diameter and mechanical strength of the material.

It was found that aging of the solution has a dramatic effect on fibre diameters as well as on mechanical strength of the material. The shape of the fibre diameter distribution graphs of samples aged for various periods (Figure 8a-c) did not change significantly. The average fibre diameter dropped from 360 nm to 300 nm after aging the electrospinning solution for 3h at room temperature (Figure 8d). Fibre diameters decrease probably because viscosity of solutions of gelatin in aqueous acetic acid decreases in time, which is probably caused by protonation of gelatin molecules. All in all, the effect of aging of the electrospinning solution is the main drawback of using aqueous acetic acid as a solvent for electrospinning gelatin and must be kept in mind during preparation and characterization of the material.

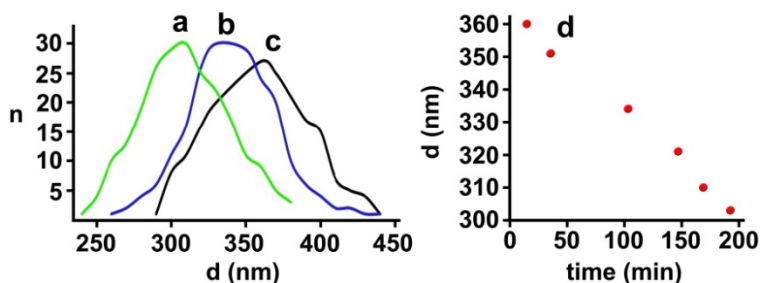


Figure 8 Fibre diameter distributions of samples aged for (a) 170 min, (b) 100 min, (c) 40 min and (d) dependence between aging time and average fibre diameter

4.4 The extent of cross-linking

4.4.1 FTIR analysis

The fabrics were cross-linked by placing them in an oven at 170–175°C. FTIR was used to determine the optimum duration of thermal treatment and to evaluate the extent of cross-linking by comparing the changes in relative absorbances brought about by varying glucose concentration and duration of

thermal treatment. Analysing the changes brought about by thermal treatment using FTIR is a challenging task complicated by the following factors. Firstly, absolute values of recorded absorbances were not reproducible due to the fibrous structure of the material and small fibre diameter, which did not allow achieving perfect contact between the fabric and ATR crystal. In order to be able to reliably compare infrared spectra of samples with different composition and analyse the changes brought about by thermal treatment, it was necessary to calculate relative absorbances as described above. Secondly, infrared absorption is greatly affected by water content in the sample. It was found that analysing spectra by calculating relative absorbances produces meaningful results only if the fabrics are properly dried prior to recording spectra. In the current work, the samples were dried in a critical point drier before analysing them. Thirdly, FTIR spectra are affected by the non-homogeneous composition of gelatin. The effect is small, but noticeable, and can be overcome by recording several spectra of every sample and averaging the absorbances.

It was concluded that FTIR can be used to analyse the cross-linking process and to determine the extent of cross-linking, given that these problems are properly addressed during experimental work. The described method cannot be used to determine the extent of cross-linking of a particular sample – it is only suitable to study the process, because it is necessary to prepare and analyse large patches of samples in order to get meaningful results.

FTIR spectra of gelatin, glucose and the fabrics before and after thermal treatment are presented in Figure 9. Peaks were detected at 3285, 3077 cm^{-1} (mainly OH and NH vibrations), 2939 and 2879 cm^{-1} (mainly CH_2 asymmetric and symmetric vibrations respectively), 1657, 1640 and 1631 cm^{-1} (the amide I band, mainly C=O vibrations), 1562, 1547 and 1535 cm^{-1} (the amide II band, mainly NH bending), 1452, 1405, 1336, 1203 and 1163 cm^{-1} (different in plane vibrations) and 1240 cm^{-1} (the amide III band, in plane NH bending and CN stretching), 1081, 1035 and 921 cm^{-1} (mainly CO vibrations overlapping with over vibrations in glucose [I, 91, 92, 93, 94]). It was found that the changes in gelatin brought about by thermal treatment of the fabrics are best represented by changes in relative absorbance of the amide I peak, whereas changes in glucose are best represented by changes in relative absorbance of a peak at 1035 cm^{-1} .

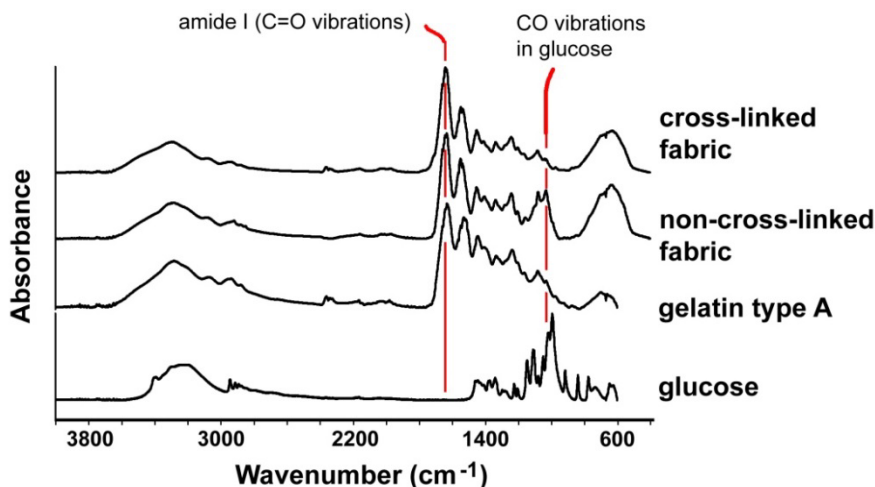


Figure 9 FTIR spectra of glucose, type A gelatin and electrospun fabrics containing 15.1% glucose before and after thermal treatment.

4.4.2 Monitoring the cross-linking process

Increasing glucose content in the fibres from 0% to 30% led to considerable increase in relative absorbance of peaks at 1081 cm^{-1} , 1035 cm^{-1} and 921 cm^{-1} . At the same time, relative absorbance of amide I and amide II bands decreased. These are the peaks by which we can best distinguish between changes in gelatin and changes in glucose brought about by thermal treatment. In order to find the optimal duration of thermal treatment, the cross-linking process was monitored by removing pieces of scaffolds from the oven after various times, analysing these by FTIR and comparing the resulting spectra. It was found that changes in relative absorbance during thermal cross-linking are exponential (Figure 10). Considerable changes in relative absorbance were detected during the first hour of cross-linking. After 2 hours of thermal treatment, very little further changes in relative absorbance were detected with an average of less than 0.1% change between 2 and 3 hours as opposed to an average of about 5% change in relative absorbance during the first 5 minutes of thermal treatment. It was concluded that maximum extent of cross-linking is reached after nearly 3 h of thermal treatment.

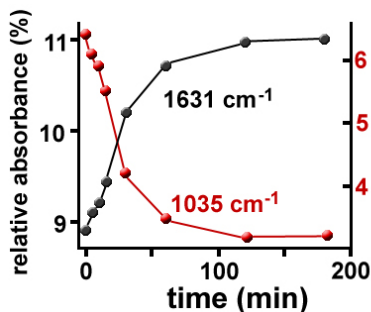


Figure 10 Changes in relative absorbance during thermal treatment of the fabrics (19.9% glucose content)

Before thermal treatment, the fabrics were water-soluble. After cross-linking, the fabrics became insoluble in boiling water and glacial acetic acid. The material also became insoluble in cell culture media [I]. Thermal treatment did not have a significant effect on the morphology of the material (Figure 11). This is an advantage compared to several other cross-linking techniques (e.g. cross-linking using glutaraldehyde vapour), which might have an effect on the morphology of the material.

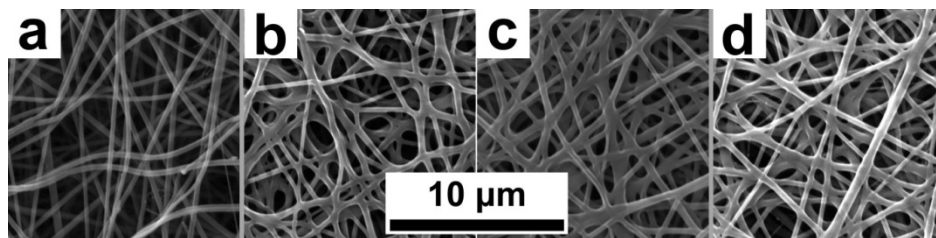


Figure 11 21000 times magnified SEM images of gelatin fabrics containing 0% (a, b) and 15.1% (c, d) glucose before (a, c) and after (b, d) thermal treatment. It can be seen that thermal treatment does not have a significant effect on the morphology of the fabrics.

4.4.3 Determination of the extent of cross-linking

FTIR analysis was used to determine maximum extent of cross-linking that can be achieved by using glucose as a cross-linking agent. Spectra of thermally treated and non-treated gelatin fabrics containing 0–30% glucose were compared to analyse structural changes caused by thermal treatment and to determine the extent of cross-linking. Major changes in relative absorbance were seen at 1647, 1640, 1631, 1081 and 1035 cm^{-1} . Changes in relative absorbance (Figure 12) were calculated by subtracting the relative absorbance of a given peak before cross-linking from relative absorbance of the same peak after cross-linking.

The interpretation of spectral changes is made harder by the number and complexity of reactions going on during cross-linking. Whereas spectral changes in pure gelatin fabrics are limited, it was observed that samples with higher glucose content undergo rapid changes during thermal treatment, which indicates that the majority of reactions going on during cross-linking are directly caused by the presence of glucose. By far the biggest change detected was an increase in relative absorbance of the amide I band, coupled with the decrease of peaks at 1081 and 1035 cm^{-1} associated with C-O vibrations mainly in glucose.

This brings up the question of the amount of glucose at which gelatin is cross-linked to maximum extent that can be achieved using glucose as cross-linking agent. The extent of gelatin cross-linking is often evaluated by changes in free ϵ -amino group concentration [95], although it has been suggested that at high temperatures a cross-linking mechanism without an amino group involvement occurs [96]. The increase in relative absorbance at 1657, 1640 and 1631 cm^{-1} was strongest for fabrics containing about 20% glucose, while relative absorbance at 1081 and 1035 cm^{-1} decreased further still when scaffolds contained more glucose. This suggests that some other reaction, perhaps caramelization, becomes more dominant at over 20% glucose content.

FTIR analysis alone does not prove that the reactions caused by thermal treatment actually lead to formation of cross-links between gelatin molecules. However, taking into account that mechanical strength of the fibre material increases with increasing glucose content [II] and that the material becomes more resistant to enzymatic degradation [I], it can be concluded that glucose does indeed act as a cross-linking agent for gelatin, and that the maximum extent of cross-linking is achieved at about 20% glucose content. However, fabric-like, easy to handle material can be obtained from gelatin-glucose blends containing up to 15% glucose. At higher glucose concentrations the fabrics become impractically brittle after thermal treatment.

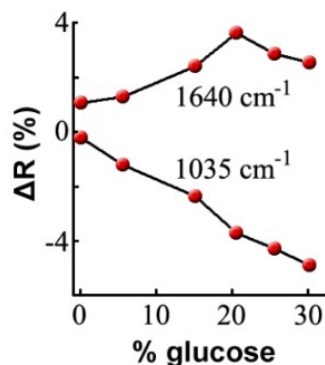


Figure 12 Changes in relative absorbance (ΔR) at 1640 cm^{-1} caused by thermal treatment indicate that maximum extent of cross-linking is achieved at about 20% glucose content. Changes in relative absorbance were calculated by subtracting relative absorbance of a given peak before cross-linking from relative absorbance of the same peak after cross-linking.

4.5 Mechanical properties of electrospun gelatin

4.5.1 Tensile test and simulation

Mechanical properties of electrospun gelatin were characterized by tensile test. Pure gelatin scaffolds showed considerable neck formation during tensile test, while the width of scaffolds containing 15% glucose did not change at all during the test (Figure 13). Force and elongation were measured during the test. A short elastic region was found to be typical (Figure 14a). Considerable elongation was seen in the final part of the diagram as the material was slowly ripped apart. Relatively long experimentally determined plastic region was not simulated, because structural changes contribute greatly to the shape of this region and the approximation of straight fibres is not valid any more, and the aim of modelling was to evaluate elastic properties of fibre material.

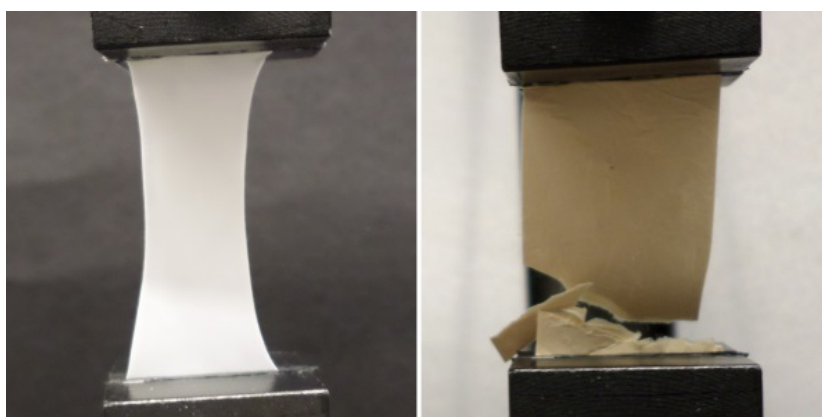


Figure 13 Neck formation during tensile test: samples containing 0% glucose (left) and 15% glucose (right).

In order to compare the influence of E (elastic modulus) and the influence of E/G ratio (where G is a coefficient which's value is proportional to the shear modulus of the fibre material) on the results, tensile test was simulated using a wide range of values of E and E/G ratio. Typical results of the calculated force-elongation diagrams are shown in Figures 14b and 14c. The applied force calculated from the model is proportional to the number of fibres in a particular scaffold. Therefore, the force is proportional to the total length of the fibres in each scaffold. The total force applied to the scaffold is also proportional to the cross-section area, since the force contributions of fibres are multiplications of stress and cross-section area $\pi d^2/4$, where d is the fibre diameter. The diameter and the total length of the fibres as well as the initial length and width of the mesh used in the calculations were determined experimentally beforehand. It can be seen (Figure 14b, c) that the calculated applied force strongly depends on

the elastic modulus of the fibre material whereas the dependence on the E/G ratio is rather weak.

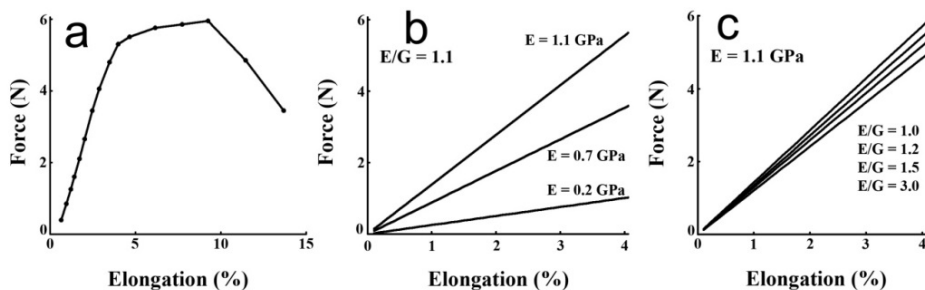


Figure 14 Experimental tensile test (a) and simulation results demonstrating the effect of elastic modulus (b) and E/G ratio (c) on force-elongation curve in the elastic region

4.5.2 Effect of glucose content

The elastic modulus of the fibre material was determined for samples prepared using 0–15% glucose. It was found (Figure 15a) that cross-linking by glucose considerably increases the elastic modulus of gelatin fibres from about 0.3 GPa (0% glucose) to 1.1 GPa (15% glucose). Simulation results were used to draw connections between the elastic modulus of the scaffold and the elastic modulus of the fibre material (Figure 15b) at different E/G ratios calculated for a wider range of values of E . The analysis revealed that the elastic modulus of the fibre material exceeds the elastic modulus of the scaffold around 8 times. These results seem to be reliable, taking into account the porosity and fibrous structure of the mesh.

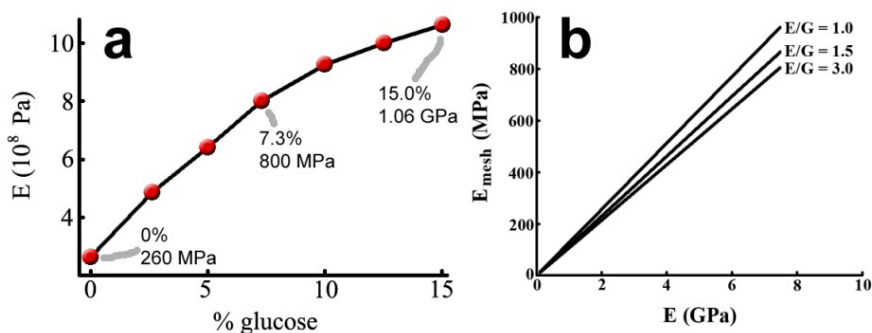


Figure 15 The effect of glucose concentration on elastic modulus of gelatin fibres (A); Dependence between elastic modulus of the fibre material and elastic modulus of the mesh (B)

4.5.3 Effect of aging of the electrospinning solution

The elastic modulus of the fabric decreased from 138 MPa to 53 MPa as the aging time of the electrospinning solution increased from 15 min to 3 h (Figure 16). Therefore, it is vital to keep the aging time constant for all samples in order to be able to reliably compare the properties of fabrics containing different amount of glucose and other additives. In the current work, aging time was kept as short as possible (below 15 minutes as a general rule).

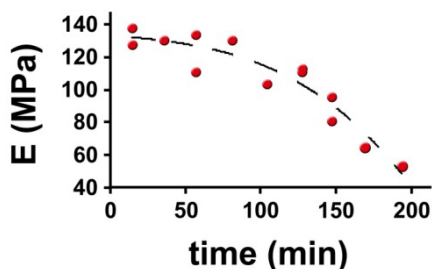


Figure 16 Aging the electrospinning solution causes a decrease in the elastic modulus of the material (experimental data given with a 3rd order polynomial trend line)

4.6 Effect of alum on electrospun gelatin cross-linked by glucose

4.6.1 Preparation of alum-containing fabrics

Additives could help us to further increase the extent of cross-linking of electrospun gelatin cross-linked by glucose. In the current study, the effect of alum (aluminium potassium sulphate) on preparation and properties of nanofibrous electrospun gelatin meshes cross-linked by glucose was investigated. $\text{AlK}(\text{SO}_4)_2$ did not have a significant effect on the fibre preparation process even at higher concentrations. Additionally, $\text{AlK}(\text{SO}_4)_2$ did not have a significant effect on the morphology of the meshes (Figure 17 a–b), except for on fibre diameters. The addition of $\text{AlK}(\text{SO}_4)_2$ brought about two changes. Firstly, average fibre diameters increased with increasing salt content. This was caused mainly by the fact that the same amount of solvent was used to prepare all the electrospinning solutions, but the concentration of the solution increased (the amount of solvent per solution volume decreased) as the salt was added. The effect of $\text{AlK}(\text{SO}_4)_2$ on average fibre diameters is hard to detect, probably because it is small compared to the effect of the changes in concentration of the electrospinning solution. Secondly, fibre diameter distribution graphs (Figure 17 c–e) revealed that the measured fibre diameters were more dispersed in case of samples containing more salt. This is probably caused by the higher electrical conductivity of the polymer solution, which in return makes the charged polymer solution less stable.

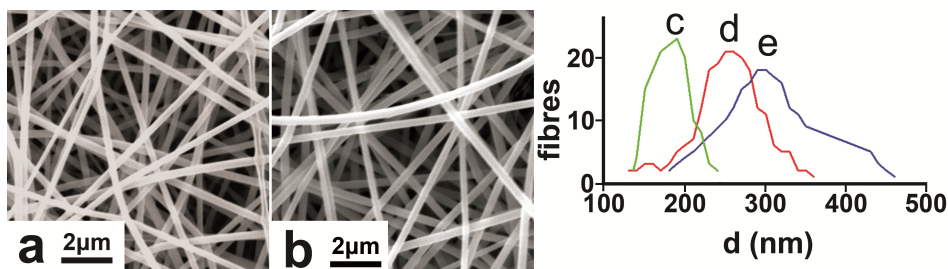


Figure 17 SEM images of gelatin meshes cross-linked by glucose (21000 times magnification) containing 0% (a), 17.1% (b) $\text{AlK}(\text{SO}_4)_2$. Fibre diameter distributions of samples containing 0% (c), 4.8% (d), 13.4% (e) $\text{AlK}(\text{SO}_4)_2$

4.6.2 Mechanical properties of alum-containing fabrics

Mechanical behaviour of alum-containing electrospun gelatin was studied by tensile test. Tensile test results and stress-strain curves are demonstrated in Figure 18. The simulation results shown on the figure correspond to the best fit of the elastic moduli of the fabrics. The main problem causing uncertainties and variabilities in stress-strain curves is the fact that the thickness of the samples is not uniform. Due to the nature of the electrospinning process, the thickness of the samples varied up to 20%. This is somewhat compensated in the model used to evaluate the elastic modulus of the fibre material by taking into account the total length of the fibre per volume, but is nevertheless a possible cause of uncertainty.

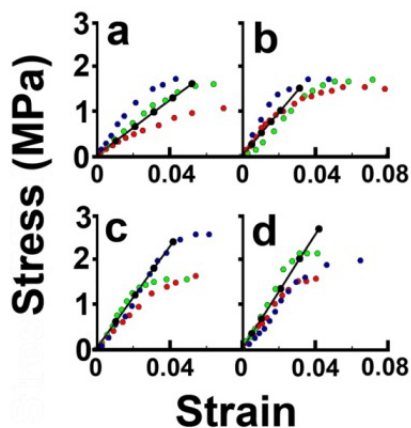


Figure 18 Tensile test results: samples without additives (a) and samples containing 3.0% (b), 5.6% (c), 8.8% (d) $\text{AlK}(\text{SO}_4)_2$ and the simulated elastic region of the stress-strain curve (lines on a–d)

As a general rule, the most important part of the stress-strain curve is the linear region, because in most applications the material only works in the elastic region of the stress-strain diagram. Therefore, plastic region and failure of the meshes were not studied. The elastic moduli of the meshes with different composition were calculated using the linear region of the stress-strain curve. Next, tensile test was simulated by varying the hypothetical elastic modulus of the fibre material, until the elastic region of experimental stress-strain curves and simulated stress-strain curves overlapped. In this case the elastic modulus of a fibrous material calculated from the simulation is equal to the experimentally determined elastic modulus of the mesh. The elastic modulus of the fibre material was found in this way for all samples.

$\text{AlK}(\text{SO}_4)_2$ increased the elastic modulus of glucose-cross-linked gelatin fibres up to about 10% salt content (Figure 19). However, it should be kept in mind that the absolute values of the determined elastic moduli are dependent on numerous factors mentioned in the introduction, including molecular mass of gelatin, extent of cross-linking determined by the cross-linking method (the amount of cross-linking agent, duration of thermal treatment, temperature), possible degradation of gelatin, surface effects, etc. In the current study, these parameters were kept as constant as possible in order to distinguish the effect of the additive from other parameters. Thereby, the effect of $\text{AlK}(\text{SO}_4)_2$ was clearly evident. In the current work, the elastic modulus of electrospun gelatin fabrics increased from 20 MPa to 70 MPa and the elastic modulus of the fibre material increased from 150 MPa to 620 MPa as the salt content increased from 0% to 9.6%. However, varying the abovementioned parameters can cause fibrous gelatin-based materials to be either mechanically stronger or weaker than hereby presented. During preparation of the fabrics, obtaining homogeneous electrospinning solutions took longer in case of gelatin mixtures containing alum (around 15 min), compared to mixtures containing only gelatin and glucose (in which case 5 min of stirring was sufficient). This is a drawback of the addition of alum, since the strength of the fibres decreases with increasing solution aging time.

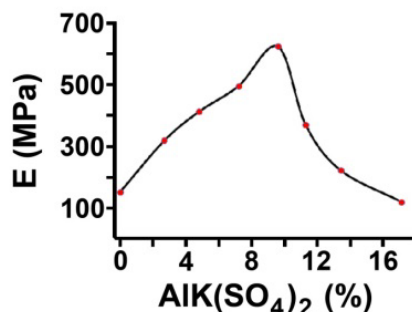


Figure 19 Dependence of the elastic modulus of the fibre material on $\text{AlK}(\text{SO}_4)_2$ concentration.

The elastic modulus of the fibre material is roughly 8 times higher than the elastic modulus of the mesh. This difference is caused firstly by the high porosity of electrospun materials and secondly by random fibre orientation. The fact that the addition of $\text{AlK}(\text{SO}_4)_2$ increased the elastic modulus of the material indicates that the presence of the salt induces cross-linking, which in return determines mechanical strength of the material. At about 10% $\text{AlK}(\text{SO}_4)_2$ content maximum extent of cross-linking that can be reached by the addition of $\text{AlK}(\text{SO}_4)_2$ is achieved and the elastic modulus of the material drops at higher salt concentrations. It is not certain whether the addition of $\text{AlK}(\text{SO}_4)_2$ brings about covalent or ionic cross-linking, but significant changes in FTIR spectra between $950\text{--}1300\text{ cm}^{-1}$ brought about by the presence of the salt, in addition to considerable increase in strength of the material indicate that thermal treatment initiates covalent cross-linking (probably involving carboxyl groups of gelatin), although the exact chemistry of it requires further studies.

5. DISCUSSION

Cross-linking of electrospun gelatin

Evaluating the extent of cross-linking by analysing FTIR spectra proved to be effective. However, it must be noted that the determined extent of cross-linking is dependent on the method used to evaluate the extent of cross-linking. Therefore, the results are hard to compare to those obtained by other methods, for example near-infrared spectrophotometry [97] and use of chemical assays [98]. FTIR analysis indicated that gelatin reacts chemically with glucose at up to 20% glucose content. At higher glucose concentrations some other kind of reaction, probably caramelization, becomes prevalent. The following additional information must be considered in order to prove that the chemical reaction between gelatin and glucose leads to the formation of cross-links between gelatin molecules. Firstly, mechanical strength of electrospun gelatin increases with increasing glucose content. This indicates that cross-links between gelatin molecules do form. However, only meshes containing up to 15% glucose were tested since the fabrics became very brittle at over 15% glucose content, and the attempts to test samples containing more glucose were not successful. Secondly, it was discovered that meshes containing up to 15% glucose were enzymatically digestible, meshes containing 20% were partially enzymatically digestible, and meshes containing over 20% glucose were totally resistant to enzymatic degradation [I]. Taking all the results into account, it can be concluded that electrospun gelatin can be thermally cross-linked by glucose and that the extent of cross-linking increases up to 20% glucose content. Compared to other widely used cross-linking agents, glucose is not only biocompatible and non-toxic, but also relatively cheap and allows producing the fabrics using only natural substances and easy, cost-effective methods.

Glucose-initiated cross-linking reaction involves mainly certain amino groups of gelatin. Non-cross-linked gelatin contains around 23×10^{-5} mol/g free amino groups [99]. Samples containing 20% glucose contain around 110×10^{-5} mol/g glucose molecules. Therefore, certain amount of caramelization probably occurs in glucose-containing gelatin meshes, which is supported by the fact that thermal treatment was carried out at over melting point and caramelization temperature of glucose. This in return indicates that the cross-links between gelatin molecules might actually be caramel molecules of varying size, which further supports the formation of cross-links.

Mechanical properties of electrospun gelatin

The model used to evaluate the elastic modulus of the fibre material in the current study describes mechanical behaviour of fibrous cross-linked or otherwise interconnected materials and allows simulating tensile test in the elastic region. Remaining in the elastic region is related to the approximation that the fibres in the concerned part of a scaffold are straight during tensile test and do not slide on each other. This assumption is only valid if there are chemical bonds present at fibre interceptions. In case of gelatin scaffolds, the fibres must be cross-

linked to make them insoluble and observations considered above indicate that chemical bonds appear between individual fibres during cross-linking. On a large scale, electrospun fibres are deposited in spirals, but considering the fibres to be straight is a good approximation because of the cross-links at fibre interceptions and the fact that the model operates in the elastic region. In any case, it is the elastic region that is of importance in most applications. However, for brittle fibre materials with no plastic deformation one may determine the maximum normal and tangential stresses to simulate the formation of stress-strain curve for a scaffold. Performing such simulations showed that failure of the fibres one by one leads to plastic deformation of a mesh.

Tensile test was simulated for a small part of a scaffold and this part was assumed to remain rectangular during the simulation. Such assumption is strictly valid for all parts of the scaffold, if the width of the sample does not change during the test. Considerable neck-formation was observed in case of pure gelatin and non-cross-linked scaffolds, leading to the conclusion that forces affecting the edges of the rectangular parts of the scaffold do not remain parallel to the force applied to the sample during the test. Friction at the sample fixation point creates a force perpendicular to the applied force. Therefore, the accuracy of the model is the better the less neck-formation is observed.

Finally, the following aspect of mechanical characterization of electrospun gelatin should be studied further in the future. Both covalent bonds (cross-links) and non-covalent bonds (especially hydrogen bonds) between gelatin molecules affect mechanical strength of the fibres. However, the quantity of these bonds is strongly influenced by the following effect. As mentioned, gelatin molecule only has a limited number of sites where cross-links can form (free amino groups of gelatin). Some of these sites are located inside the fibres and others on the surface of the fibres. The proportion of the cross-linking sites located on the surface of the fibre increases as the fibre diameter decreases. On the surface, cross-links can only be formed between individual fibres (not between molecules inside an individual fibre), but the probability for this is low due to the high porosity of electrospun materials. Such surface effect should become more dominant as the fibre diameter decreases due to increasing specific surface area and the characteristics of the fibres become more distorted compared to bulk material. Hydrogen bonding and other non-covalent forces present in protein fibres also have a similar effect on mechanical strength of the fibres. The formation of covalent cross-links can be expected to decrease the importance of the role of hydrogen bonds and other non-covalent forces in determining the strength of gelatin fibres. Such an effect should contribute more to the strength of larger fibres.

6. APPLICATIONS – TISSUE ENGINEERING

Tissue engineering is an interdisciplinary field of research, which unites materials science, engineering, chemistry, physics, medicine and cell biology. In the current study [I], electrospun gelatin meshes thermally cross-linked by glucose were used as cell culture scaffolds to evaluate their suitability for tissue engineering applications. In order to do that, fibroblasts were seeded onto glass cover slips covered with electrospun gelatin scaffolds [I]. Cells grown on electrospun gelatin are compared to cells grown on glass in Figure 20.

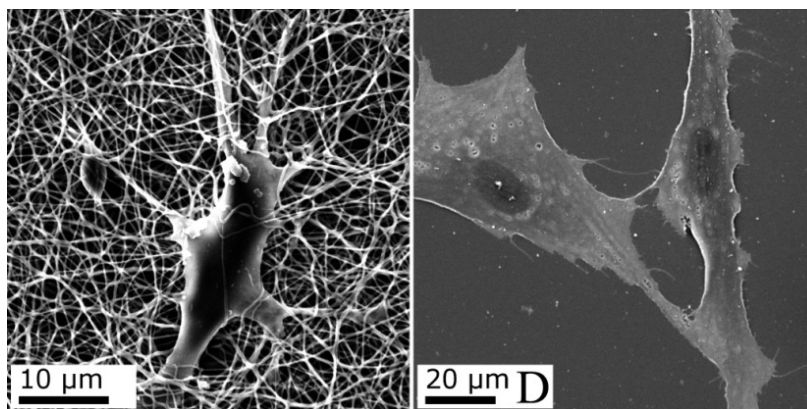


Figure 20. SEM images of cells grown on electrospun gelatin (left) and glass (right [I]).

Morphological differences between cells grown on glass and cells grown on the scaffolds can be explained in the following way. Natural extracellular matrix consists of bundled nanofibres. In order to mimic this structure, artificial extracellular matrix should also consist of fibres with sub-micron diameters. Electrospinning has the advantage of producing structural features somewhat similar to native extracellular matrix, which has been shown to result in different cell morphology compared to cells grown on smooth substrate [100].

Cell culture experiments, biological stability test and observations made during scaffold preparation [I] indicate that scaffolds containing up to 15% glucose can be considered for tissue engineering applications, most importantly because fabric-like easy to handle scaffolds were produced at 0–15% glucose content, secondly because the number of cells on scaffolds started to drop at higher glucose concentrations, and thirdly because at over 15% glucose content the scaffolds become resistant to enzymatic degradation.

7. CONCLUSIONS

The following conclusions were made based on the current study.

Gelatin was electrospun from 10M aqueous acetic acid. Average fibre diameter varies from 200 nm to 700 nm depending on the concentration of the electrospinning solution. FTIR analysis, results of mechanical characterization of the material, biological degradation tests, and observations made during the experiments indicate that electrospun gelatin can be thermally cross-linked by glucose by placing the fabrics in an oven at about 170°C.

Maximum extent of cross-linking is achieved at nearly 20% glucose content. However, easy to handle fabrics can be obtained from gelatin-glucose blends containing up to 15% glucose. At higher glucose concentrations the material becomes impractically brittle after thermal treatment. The extent of cross-linking depends on glucose concentration and duration of thermal treatment. Maximum extent of cross-linking is reached after nearly 3 h of thermal treatment.

Mechanical properties of the material were evaluated by tensile test and modelling. The model simulates deposition of fibres during electrospinning and tensile test of fibrous meshes in order to evaluate the elastic modulus of electrospun materials and is effective when applied to cross-linked fabrics with bonds between both polymer molecules and at fibre interceptions. Cross-linking electrospun gelatin by glucose increases mechanical strength of the material. The addition of aluminium potassium sulphate further increases mechanical strength of electrospun gelatin cross-linked by glucose up to about 10% alum content. Aging of the electrospinning solution decreases mechanical strength and fibre diameter of gelatin electrospun from 10M aqueous acetic acid.

Preliminary short-term cell culture experiments indicate that electrospun gelatin cross-linked thermally by glucose is suitable for tissue engineering applications [I].

All in all, the goals of the current work were fully achieved and a promising new material, electrospun gelatin cross-linked by glucose, was developed as a result of the work.

SUMMARY IN ESTONIAN

Glükoosiga ristsidestatud elektrospinnitud želatiin

Käesolevas doktoritöö algseks eesmärgiks oli siirdamiseks sobivate želatiini-põhiste naharakkude kasvualuste väljatöötamine, kasutades võimalikult loodus-sõbralikke lähteaineid ja meetodeid. Želatiin valiti peamiseks lähteaineks, kuna see on äärmiselt biosobiv, mitte-toksiline, kergesti töödeldav ja odav. Želatiini saadakse kollageeni, ühe sidekoe peamise koostisosa, hüdrolyüsi tulemusel. Kollageeni ja želatiini keemiline koostis on seetõttu peaaegu identne, mis teeb želatiini eriti sobivaks lähteaineks naharakkude kasvualuste valmistamisel. Želatiini peamiseks puuduseks võrreldes sünteetiliste lähteainetega on mitte-homogeenne koostis ja sellest tulenev füüsikaliste ja keemiliste omaduste varieeruvus, kuid antud juhul on eeliseid peetud tunduvalt kaalukamaks puudustest.

Elusorganismis koosneb normaalne rakuväline keskkond põhimõtteliselt kokkukeerdunud nanokiudude pundardest. Sellise struktuuri teatud ulatuses jäljendamiseks valmistati kasvualused elektrospinnimise meetodil. Elektrospinnitud želatiin on aga vees lahustuv ja mehaaniliselt nõrk. Need puudused on võimalik ületada želatiinimolekulide ristsidestamise teel. Varasemalt on selleks otstarbeks kasutatud mitmeid kalleid ja vähem või rohkem toksilisi keemilisi ristsidestajaid. Käesolev dissertatsioon põhineb avastusel, et elektrospinnitud želatiini on võimalik ristsidestada glükoosi abil. See avastus tekitas mitmeid küsimusi – kui palju glükoosi peaks želatiinile lisama soovitud tulemuse saamiseks, kui kaua peaks želatiinikangast kuumutama, kuidas ristsidestamine mõjutab materjali omadusi, kas glükoosiga ristsidestatud želatiinikangas on sobiv regeneratiivses meditsiinis kasutamiseks jne. Käesolev dissertatsioon koos selle aluseks olevate artiklitega [I–III] annab vastuse mitmetele praktilistele küsimustele.

Suur osa dissertatsioonist on pühendatud glükoosiga ristsidestatud elektrospinnitud želatiinikanga mehaanilistele omadustele, kuna just need mängivad võtmerolli paljudes rakendustes, sealhulgas biomeditsiinis. Samas on just želatiini mehaaniline nõrkus olnud tema laiema kasutamise üheks peamiseks takistuseks ja on väga oluline keskenduda nende meetodite arendamisele, mis aitaksid želatiinikiude tugevamaks muuta. Lisaks on biopolümeersete kiudude mehaanilised omadused küllaltki kompleksed ja mõjutatud muuhulgas lähteaineks oleva želatiini kvaliteedi, valmistamisprotsessi parameetrite, lahusti omaduste, niiskuse, kiu läbimõõdu jms poolt. Kõigi nende mõjurite tõttu varieerub želatiinikiudude kirjanduses toodud elastsusmoodul äärmiselt suures ulatuses, kirjanduses leiduvaid tulemusi on omavahel väga raske võrrelda ja katsete korratavus ei ole hea, mis tekitab palju segadust. Seega on äärmiselt tähtis uurida elektrospinnitud želatiini mehaanilisi omadusi piisavalt suure detailsuse astmega, et oleks võimalik korratavalt valmistada kindlate mehaaniliste omadustega kangaid. Eeltoodust tulenevalt on käesoleva uurimuse

peamiseks eesmärgiks valmistada ja iseloomustada elektrospinnitud glükoosiga termiliselt ristsidestatud želatiinikangaid.

Töö tulemusena leiti, et želatiini ning želatiini, glükoosi ja teiste lisandite segusid on võimalik elektrospinnida 10 M äädikhappe vesilahusest. Keskmised kiudude läbimõõdud jäävad vahemikku 200–700 nm olenevalt želatiini ja lisandite kontsentratsioonist elektrospinnimise lahuses. Kangataolist, kergesti käsitletavat materjali on võimalik saada kuni 15% glükoosi sisaldavatest segu-dest. Suurema glükoosisisalduse juures muutub materjal pärast kuumutamist väga hapraks.

FTIR analüüs, želatiinikanga mehaaniliste omaduste määramise katsete tulemused, lagundamiskatsed ja katsete käigus tehtud tähelepanekud kinnitasid seda, et glükoos tõepoolest ristsidestab kiude moodustavaid želatiinimolekule, kui ta paigutada ahju umbes 170 °C juures. Seeläbi muutub želatiinikangas lahustumatuks ja mehaaniliselt palju tugevamaks. Ristsidestumise määr sõltub glükoosikontsentratsioonist ja kuumutamise kestvusest. Maksimaalne ristsidestumise ulatus saavutatakse umbes 3 tunni kuumas ahjus hoidmise järel.

Želatiinikanga mehaanilisi omadusi iseloomustati tõmbekatse ja modelleerimise teel. Mudel simuleerib esiteks kiudude kogunemist elektrospinnimise ajal, teiseks kiududest koosneva kanga tõmbekatset eesmärgiga määrata kiumaterjali elastsusmoodul. Mudel on eriti sobiv rakendamiseks ristsidestatud kiudmaterjalidele, mille puhul on keemilised sidemed mitte üksnes kiude moodustavate želatiinimolekulide vahel, vaid seotud on ka kiud omavahel kiudude ristumiskohtades. Leiti, et glükoosiga ristsidestamine suurendab oluliselt elektrospinnitud želatiini elastsusmoodulit. $\text{AlK}(\text{SO}_4)_2$ lisamine muudab glükoosiga ristsidestatud elektrospinnitud želatiini mehaaniliselt veelgi tugevamaks. Samas elektrospinnimise lahuse vanandamine vähendab valmistatava kanga mehaanilist tugevust, kui lahustiks on äädikhappe vesilahus.

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Education

2012–... PhD student* (Materials Science, University of Tartu, Estonia)
2012 MSc in Engineering (Materials Science), *cum laude* (University of Tartu, Estonia)
2008 MA in Law (University of Tartu, Estonia)

* project 'Elaboration of polymer nanofiber-based nano- and microstructural materials'

Professional summary

2012–2015 University of Tartu, Institute of Physics, Laboratory of Physics of Nanostructures (specialist/engineer)*
2008–2012 Breyra LLC (member of board)
2007–2008 Estonian Ministry of the Interior, Internal Security Policy Department (acting legal adviser)
2006–2007 Estonian Tax and Customs Board (lawyer)

* projects „Elaboration of polymer nanofiber-based nano- and microstructural materials,, and „Smart aerogels based on the nanostructured wood cellulose“

Teaching experience

2012–2015 Practical Works in Materials Physics II. Electricity and Magnetism (Faculty of Science and Technology/ Institute of Physics, University of Tartu)

Publications

1. Kaido Siimon, Paula Reemann, Annika Pöder, Martin Pook, Triin Kangur, Külli Kingo, Viljar Jaks, Uno Mäeorg, Martin Järvekülg. Effect of glucose content on thermally cross-linked fibrous gelatin scaffolds for tissue engineering. *Materials Science & Engineering C* (2014) 42: 538–545.
<http://dx.doi.org/10.1016/j.msec.2014.05.075>
2. Kaido Siimon, Hele Siimon, Martin Järvekülg. Mechanical characterization of electrospun gelatin scaffolds cross-linked by glucose. *Journal of Materials Science: Materials in Medicine* (2015) 26:37.
<http://dx.doi.org/10.1007/s10856-014-5375-1>

3. Kaido Siimon, Karol Mõisavald, Hele Siimon, Martin Järvekülg. Increasing mechanical strength of electrospun gelatin nanofibers by the addition of aluminum potassium sulfate. *Journal of Applied Polymer Science* (2015), 132 (35): 42431.
<http://dx.doi.org/10.1002/app.42431>

Awards

2015	Memorial Stipend of Valda and Bernard Õun
2016	Outstanding Reviewer (Materials Science & Engineering C)

ELULOOKIRJELDUS

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Haridus

2012–... doktorant* (materjaliteadus, Tartu Ülikool, Eesti)
2012 MSc (materjaliteadus), *cum laude* (Tartu Ülikool, Eesti)
2008 MA (Tartu Ülikool, Eesti)

* projekt 'Polümeersetel nanofiibritel põhinevate nano- ja mikrostruktuursete materjalide arendamine'

Töö

2012–2015 Tartu ülikool, füüsika instituut, nanostruktuuride füüsika labor (spetsialist/insener)*
2008–2012 Breyra OÜ (juhatuse liige)
2007–2008 Siseministeerium, sisejulgeolekupoliitika osakond (õigusnõuniku kt)
2006–2007 Maksu- ja Tolliamet (jurist)

* projektid „Elaboration of polymer nanofiber-based nano- and microstructural materials,, and „Smart aerogels based on the nanostructured wood cellulose“

Õpetamiskogemus

2012–2015 Materjalifüüsika praktikum II. Elekter ja magnetism (Tartu Ülikool, Füüsika Instituut)

Publikatsioonid

1. Kaido Siimon, Paula Reemann, Annika Pöder, Martin Pook, Triin Kangur, Külli Kingo, Viljar Jaks, Uno Mäeorg, Martin Järvekülg. Effect of glucose content on thermally cross-linked fibrous gelatin scaffolds for tissue engineering. *Materials Science & Engineering C* (2014) 42: 538–545.
<http://dx.doi.org/10.1016/j.msec.2014.05.075>
2. Kaido Siimon, Hele Siimon, Martin Järvekülg. Mechanical characterization of electrospun gelatin scaffolds cross-linked by glucose. *Journal of Materials Science: Materials in Medicine* (2015) 26:37.
<http://dx.doi.org/10.1007/s10856-014-5375-1>

3. Kaido Siimon, Karol Mõisavald, Hele Siimon, Martin Järvekülg. Increasing mechanical strength of electrospun gelatin nanofibers by the addition of aluminum potassium sulfate. Journal of Applied Polymer Science (2015), 132 (35): 42431.
<http://dx.doi.org/10.1002/app.42431>

Auhinnad

2015	Valda ja Bernard Õuna mälestusstipendium (biomeditsiinalase teadustöö eest)
2016	Outstanding Reviewer (Materials Science & Engineering C)

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